HHS Public Access

Author manuscript

Exp Hematol. Author manuscript; available in PMC 2023 October 01.

Published in final edited form as:

Exp Hematol. 2022 October ; 114: 22–32. doi:10.1016/j.exphem.2022.08.001.

An *in vivo* model for elucidating the role of an erythroid-specific isoform of nuclear export protein Exportin 7 (Xpo7) in murine erythropoiesis

Susree Modepalli[1], **Sandra Martinez-Morilla**[2], **Srividhya Venkatesan**[2], **James Fasano**[2], **Katerina Paulsen**[3], **Dirk Görlich**[3], **Shilpa Hattangadi**[4,*], **Gary M. Kupfer**[1,*]

[1]Department of Molecular-Oncology, Georgetown University, Washington DC, DC, USA

[2]Department of Pediatric Hematology-Oncology, Yale School of Medicine, New Haven, CT, USA

[3]Department of Cellular Logistics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

[4]Division of Kidney, Urologic, and Hematologic Diseases, National Institute of Health, Bethesda, MD, USA

Abstract

Erythroid nuclear condensation is a complex process where compaction to one-tenth its original size occurs in an active nucleus simultaneously undergoing transcription and cell division. We previously found that the nuclear exportin Exportin7 (Xpo7), which is erythroid-specific and highly induced during terminal erythropoiesis, facilitates nuclear condensation. We also identified a previously unannotated, erythroid-specific isoform of Xpo7 (Xpo7B) containing a novel first exon Xpo7–1b expressed only in late Ter119+ erythroblasts. To better understand the functional difference between the erythroid Xpo7B isoform and the ubiquitous isoform (Xpo7A) containing the original first exon Xpo7–1a, we created gene-targeted mouse models lacking either exons Xpo7–1a, Xpo7–1b, or both exons 4–5, which are completely null for Xpo7 expression. We found that deficiency in Xpo7A does not affect steady-state nor stress erythropoiesis. In contrast, mice lacking the erythroid isoform, Xpo7B, display a mild anemia as well as altered stress erythropoiesis. Complete Xpo7 deficiency resulted in partially penetrant embryonic lethality at the stage when definitive erythropoiesis is prominent in the fetal liver. Inducible complete knockdown of Xpo7 confirms that both steady-state and stress erythropoiesis are affected. We also observe that Xpo7 deficiency downregulates the expression of important stress response factors, such as

*Corresponding Author: Gary M. Kupfer, MD, Gary.Kupfer@georgetown.edu, Department of Oncology, NRB W401, Lombardi Comprehensive Cancer Center, Georgetown University, 3800 Reservoir Rd, Washington, DC 20001, 203-494-0510, Co-Correspondence: Shilpa M. Hattangadi, MD, shilpam.hattangadi@nih.gov, Division of Kidney, Urologic, and Hematologic Diseases, National Institute of Health, 9000 Rockville Pike, Bethesda, MD 20892, 301-827-7442.

Authorship Contribution

S.M.M., S.M., G.M.K. designed experiments, performed research, analyzed data, and wrote and revised the manuscript; S.V., J.F., K.P., and D.G. performed experiments; S.H. designed and performed experiments, and wrote and revised the manuscript.

Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

Gdf15 and Smad3. We conclude that the erythroid-specific isoform of Xpo7 is important for both steady-state as well as stress erythropoiesis in mice.

Keywords

Anemia; Erythroid; Exportin; Fetal liver; Stress erythropoiesis

Introduction

Terminal differentiation of erythroid progenitors into mature red blood cells is a complex and highly regulated process including the survival of committed progenitors, a set number of cell divisions leading to terminal differentiation, the accumulation of hemoglobin and other erythroid-specific proteins, and global nuclear condensation culminating in enucleation only in mammals[1]. Multiple cellular pathways have been implicated in the regulation of terminal erythroid differentiation and enucleation[2]. Several new players involved in such regulatory and signaling pathways have since been identified that control these crucial cellular processes [3–11]. As important as these genes are for normal and steady state erythropoiesis, their role in and/or connection to the molecules involved in stress erythropoiesis remains an avenue of open investigation.

In steady-state erythropoiesis, the normal level of hematocrit is maintained by a regulated production of RBCs. However, under certain physiological or clinical conditions resulting in hypoxia, a systemic “stress” response is elicited to increase RBC production. “Stress erythropoiesis” is a systemic compensatory response to tissue hypoxia due to anemia. It promotes recovery from anemia by stimulating bone marrow and other extramedullary organs like the spleen and liver to increase erythropoietic rate [12, 13]. The different course of differentiation results in release of more immature erythroid cells into circulation. In mice, stress erythropoiesis occurs primarily in the adult spleen [14, 15] which acts as an extramedullary organ for erythropoiesis, and in the fetal liver [16]. Among regulators of stress erythropoiesis, GDF15 has been recently shown to be crucial in regulating expression of important genes, with *Gdf15*−/− mice also showing delayed recovery from hemolytic anemia [17]. A deeper functional understanding of how regulation of such signaling molecules influences stress erythropoiesis can have potential clinical implications for diseases associated with ineffective erythropoiesis.

Our group has found that the nuclear export protein Exportin 7 (Xpo7) is involved in nuclear condensation and enucleation during terminal erythroid maturation[18]. Xpo7 is a member of the importin-B superfamily, is erythroid-specific, is highly induced during terminal erythropoiesis, and is the only importin-B family member remaining during terminal erythroid development. Knockdown of Xpo7 in murine fetal liver erythroid progenitors did not affect important processes of erythroid development, such as production of hemoglobin or acquisition of cell surface glycophorin Ter119 but severely inhibited nuclear condensation and enucleation. Additionally, we found an unannotated erythroid-specific first exon (1b) of Xpo7 expressed in Ter119+ erythroblasts but not in earlier erythroid progenitors, whereas the ubiquitous exon 1a of Xpo7 is expressed at modest levels in many tissues[18]. In our

recent findings, we have also shown that mir181a regulates erythroid nuclear condensation by regulating Xpo7 expression[19]. In this study, we investigated the function of Xpo7 *in vivo* by characterizing the phenotype of transgenic mouse models that lacked the ubiquitous exon Xpo7–1a, the erythroid-specific exon Xpo7–1b, or that were completely null for Xpo7 expression. Based on the phenotype of these mouse models, we find that the erythroid-specific isoform of Xpo7 is important for both steady-state as well as stress erythropoiesis in mice.

Methods

Animals

Three different strains were used in this study (Figure 1). Both *Xpo7A*$^{-/-}$ (A KO) and *Xpo7 A*$^{-/-}$*B*$^{-/-}$ (AB KO) strains were generated by the Görlich Lab, according to the German animal welfare law, and bred at Yale University (New Haven, CT, US).

Briefly, *Xpo7 A*$^{-/-}$*B*$^{-/-}$ (AB KO) mice were generated by the Görlich Lab using the mouse cell line Xpo7tm1a, clone G09 (B6N.129SvExp7KO(loxP)tmDG, crossed with Flp-recombinase in Rosa26 locus (R-Flp.B6N, #003946 Jax Stock) and then with the CMV-Cre strain[20]. *Xpo7 A*$^{-/-}$ (A KO) mice were generated by the Görlich Lab using the mouse cell line B6N;129SvExp7KO(loxP)tmDG, created from targeted Sv129 MPI-ES cells, mixed background Sv129/B6N. Afterwards, the A$^{fl/fl}$ and A$^{fl/fl}$B$^{fl/fl}$ floxed strains were crossed with CMV-Cre mouse. *Xpo7 B*$^{-/-}$ (B KO) mice were generated at Yale after breeding a floxed exon 1b mouse produced in collaboration with the Yale Genome Editing Center, using CRISPR/Cas9 system with a constitutive beta-Actin Cre mouse (B6.FVB/N-Tg(ACTB-cre)2Mrt/J), purchased from the same institution. Inducible *Xpo7 A*$^{-/-}$*B*$^{-/-}$ (AB KO) mice were generated by breeding the *Xpo7-AB*$^{fl/fl}$ floxed mouse (from Görlich Lab, Germany) described above, with the inducible R26-CreERT2 mouse strain[21] (purchased from Jackson Laboratory, Ref.008463). The *Xpo7-AB*$^{fl/fl}$/R26-CreERT2/+ mouse has exons 4 and 5 floxed and expresses Cre-recombinase upon tamoxifen treatment. Mice at 4–5 weeks of age were injected with 1mg/day of tamoxifen (prepared in corn oil at a concentration of 5 mg/mL) on 5 consecutive days. Since the RBC turnover period in mice is about 42 days, analysis of hematological parameters was performed 6 weeks after tamoxifen administration.

Mice and embryos were genotyped by polymerase chain reaction (PCR) using the primers the following primers: for *Xpo7A* KO mice, A-P1 (5’-gcaggtatcccaggtaggaggtctt-3’), A-P2 (5’-cgccattttgctccattcatgctcctc-3’) and A-P3 (5’-tcccggcctgtgttattctataaggacc-3’); for Xpo7B KO mice, B-P1 (5’-ctggatgtcactagactcgc-3’), B-P2 (5’-gtagctggcagtcaggagaa-3’), B-P3 (5’-aagcaccactggggaaatgc-3’); for *Xpo7 AB* KO mice, AB-P1 (5’-gatgagggcttgagtcacgaa-3’), AB-P2 (5’-acattatttagccttgtggga-3’) and AB-P3 (5’-agaaacaataaagcccaagca-3’) (shown in figure 1); for inducible *Xpo7AB* KO mice, two additional PCRs had to be performed: one to detect the LoxP site between exons 4 and 5 (Fw-Frt-AB2: 5’-cagctgggcaagcttacagt-3’; Rv-Frt-AB2: 5’-caccactgcccaactctttt-3’), and another to detect the Cre gene in heterozygous (Cre/+) or homozygous (cre/cre) conditions (Common-Fw: 5’-aagggagctgcagtggagta-3’; Cre-Rv: 5’-cggttattcaacttgcacca-3’; WT-Rv: 5’-ccgaaaatctgtgggaagtc-3’).

Timed matings of appropriate genotype intercrosses for each strain were performed to obtain embryos at each developmental stage. Control mice used for each strain were wild type, nonfloxed, littermate mice with Cre/+ genotype (i.e., *Xpo7-A*$^{wt/wt}$/CMV-Cre /+, *Xpo7-B*$^{wt/wt}$/β-Actin-Cre/+, *Xpo7-AB*$^{wt/wt}$/CMV-Cre /+, *Xpo7- AB*$^{wt/wt}$/ R26-CreERT2/+, for *Xpo7A* KO, *Xpo7B* KO, and *Xpo7AB* constitutive and inducible knockout strains, respectively. All animal studies were approved by the Yale University Institutional Animal Use and Care Committee.

Analysis of Xpo7AB-KO mutant embryos

Couples were set up on day 0 and on day 1, and males were then removed from the cage. That day was considered 0.5 dpc in the timing of embryo collection. Embryos were dissected at multiple timepoints during development. DNA was isolated from embryonic tissues following manufacturer protocol (Qiagen), and PCR was performed to determine genotype using the primers described above. For terminal erythroid differentiation analysis, total cells from fetal livers at specific timepoints were isolated, disrupted, washed, and resuspended in PBS with 2% FBS for further analysis.

In vivo *mouse analysis and phenylhydrazine experiments*

After genotyping, whole blood samples from 4–5-week-old male mice were collected for CBC (complete blood count) evaluation by Hemavet analyzer (Drew Scientific). Data for all hematological parameters shown are from mice of same sex (male) and similar age groups. The parameters for WT mice correlate well with published data [22, 23] for C57BL/6J male mice. For stress erythropoiesis studies, 9 week-old male mice were injected with phenylhydrazine (PHZ, Sigma) (60mg/kg/day) on day 0 and 1 in order to induce acute hemolytic anemia[24, 25]. In the case of B KO mice and inducible AB KO mice, higher doses of PHZ induced lethality and hence a single dose of 80mg/kg/day was injected on day 0. Whole blood samples were collected on day 0, 3, and 7, and CBCs were measured by Hemavet analyzer. Blood smears were prepared by spreading a drop of blood across a slide. After air-drying, cells were fixed by absolute methanol and subsequently air-dried. Slides were stained with the Wright-Giemsa stain (Sigma) and viewed using an Olympus IX71 Microscope (magnification 64X). To test recovery from severe hemolytic anemia, 9–10 week-old Xpo7B KO and inducible AB KO mice were injected with a single dose of 100 mg/kg/day of PHZ. Whole blood samples were collected on day 0, 3, 7, 10 and 14, and CBCs were measured by Hemavet analyzer. Survival was tracked during the period of recovery.

Erythroid progenitor isolation and culture

Total fetal liver cells (FLC) were isolated from E13.5 embryos (WT, KO) of A and B strains. TER119-negative erythroid progenitors were separated using magnetic beads as previously described [26] and cultured for 48 hours in erythropoietin (Epo)-containing medium as previously published [27].

Immunoblotting

Protein was isolated from peripheral blood cells using RIPA cell lysis buffer. Protein samples were run on 4–12% SDS-PAGE and transferred to a nitrocellulose membrane. The primary antibodies used were the following: anti-XPO7 (generated by Görlich lab), anti-CRM1 (Santa Cruz antibody sc-5595) and anti-GAPDH (Santa Cruz antibody sc-32233). Each of the primary antibodies were prepared at 1:1000 dilution in 0.5% milk+PBST. The respective secondary antibodies used were anti-rabbit:HRP to detect XPO7 and CRM1, and anti-mouse:HRP to detect GAPDH.

FACS analysis

For FLC culture, cells were cultured for 48 hours and immunostained with APC-conjugated anti-TER119 antibody at 1:200 (BD Pharmingen) and 10 μg/ml Hoechst 33342 (Sigma) for 15 min at 37ºC in the dark. Propidium iodide (Sigma) was added to exclude dead cells from analysis.

For embryonic lethality studies, FLC were stained with APC-conjugated anti-TER119 and PE-conjugated anti-CD71 or PE-conjugated anti-CD44 antibodies, each at 1:200 (BD Pharmingen), and analyzed as previously published [28, 29] (Supp Figure S4). Cells were also stained with anti-c-kit for erythroid progenitor quantification as previously described [30].

All cytometry was performed on a BD LSRII flow cytometer (Becton Dickinson), and the samples were analyzed using BD FACSDiva and FlowJo software.

RNA Isolation and RT-qPCR

Total RNA from mouse bone marrow was extracted using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD) according to the manufacturer's instructions and normalized to cell number. First-strand cDNA was synthesized from total RNA using Super Script II first-strand synthesis system (Invitrogen). Polymerase chain reaction (PCR) primers for *Gdf15, Smad3, Xpo7,* and *Hprt1* were obtained from Keck Oligonucleotides (Yale University, USA). The sequences of the primers are listed in supplementary Table 2. The threshold cycle (Ct) value for each gene was normalized to the Ct value of hypoxanthine phosphoribosyltransferase-1 (Hprt1) and relative to the WT control. The normalized relative mRNA expression was calculated as described previously [31].

Statistical methods

Differences between groups were assessed using Student's two-tailed *t*-test or two-way ANOVA. When specified, Chi-square test for multiple groups was utilized to evaluate differences between groups. Data processing and statistical analyses were performed with the Prism software (GraphPad Software Inc.). Differences were considered significant when $p < 0.05$. Results are presented as mean ± SEM of at least 3 independent experiments or ≥ 5 animals per group.

Results

The ubiquitous isoform of Xpo7 *gene, Xpo7A, is not essential for normal erythropoiesis*

By RNAseq, we have previously described an erythroid-specific first exon of *Xpo7* (exon 1b) with a potential specific promoter that harbors sites for erythroid master regulators such as GATA1 and TAL1 [18], as well as unique 3' and 5' UTRs [19], compared to the first exon of the ubiquitous isoform, which we call Xpo7A.

To determine whether or not variants A and B of *Xpo7* have different functions *in vivo*, we evaluated three gene-targeted strains of Xpo7 mice (Figure 1), lacking the A variant (*Xpo7A* KO), the B variant (*Xpo7B* KO), or both variants (the *Xpo7AB* KO). *Xpo7A* KO mice were born in normal Mendelian ratios and survived throughout adulthood without increased rates of mortality (Table 1). At a morphological level, both KO and control mice displayed normal healthy RBC in blood smears (Figure 2A). Xpo7 loss in the testis was highly specific as compared to CRM1 (Xpo1) (Figure 2B). As expected, analysis of adult hematopoiesis in *Xpo7A* KO mice did not show any defect compared to WT mice, and spleens were normal size at baseline (Figure 2C and Supplemental Table S1). We hypothesized that these animals do not show anemia because they still express the erythroid isoform of Xpo7 (Xpo7B) in red cells.

In order to confirm that Xpo7A was not involved in stress erythropoiesis, *Xpo7A* KO mice were treated with phenylhydrazine (PHZ), which induces hemolytic acute anemia [32, 33]. We compared each mouse to itself under each category after PHZ treatment. RBC and HCT were decreased after PHZ treatment with no difference between WT and *Xpo7A* KO mice, with the lowest value recorded on day 3 (Figure 2D). As expected, RDW was increased during anemia induction on day 3. By day 7, all parameters returned to normal values similar to those on day 0. When KO mice were compared to WT, there was no significant difference in RBC and HCT, but KO animals showed a significantly higher level of RDW ($p<0.001$, two-way ANOVA) than WT after PHZ treatment. We further characterized stress erythropoiesis in these mice by analyzing FLC cultures. The number of erythroid progenitors (Ter119-negative cells) isolated from each embryo was similar for both WT and KO mice. After 48h in culture, there were no statistically significant differences in cell number or differentiation, as evident from degree of enucleation (Figure 2E).

Xpo7A knockout mice were born in normal Mendelian ratios, showed no anemia in adults, and had no impaired response to stress erythropoiesis after phenylhydrazine treatment or in FLC cultures. We hypothesize that Xpo7A does not affect steady-state nor stress erythropoiesis because *Xpo7A* KO mice still express the erythroid isoform of Xpo7 (Xpo7B) in erythroid progenitors, so next we evaluated *Xpo7B* KO mice.

Mice lacking the erythroid isoform of Xpo7 gene, Xpo7B, show a mild anemia and altered stress erythropoiesis

To determine whether the erythroid variant of *Xpo7* (*Xpo7B*) affected steady-state or stress erythropoiesis, we next generated a mouse model where only *Xpo7B* was not expressed. We hypothesized that only *Xpo7B* and not *Xpo7A* would be important for steady-state and stress erythropoiesis *in vivo*.

Xpo7B KO mice were born in a normal Mendelian distribution as a result of heterozygous intercrosses (Table 1) and displayed morphologically normal RBC (Figure 3A). Xpo7B loss in peripheral blood was complete (Figure 3B). When hematological parameters were analyzed, Xpo7B KO mice showed mild anemia compared to controls with statistically lower RBC, lower HCT, higher RDW, and increased reticulocytosis (Figure 3C, **** = $p<0.0001$, ** = $p<0.001$, * = $p<0.01$, two-way ANOVA), but normal-sized spleens. To assess recovery from severe hemolytic anemia, we injected 9–10 week-old mice with a single dose of 100 mg/kg/day of PHZ and compared each mouse to itself under each category after PHZ treatment. We found that the KO animals survived only through day 3 post treatment (data not shown). As a result, for further investigations, mice were treated with a single lower dose (80mg/kg) of PHZ. The differences in hematological parameters were stable after PHZ treatment for only the RDW (Figure 3D). However, erythroid progenitors from FLC of Xpo7B KO mice were considerably higher in number compared to controls and proliferated less in culture than controls (Figure 3E). Nevertheless, differentiation was not affected as the levels of erythroid marker Ter119 (*data not shown*) and enucleation were similar for both KO and WT cells at 48h (Figure 3E).

In summary, *Xpo7B* knockout mice were born in normal Mendelian ratios, but adults showed mild anemia. Knockouts had no impaired response to stress erythropoiesis after phenylhydrazine treatment compared to controls, but there were increased erythroid progenitors and less cell growth in FLC cultures from KO animals. We hypothesize that Xpo7B mildly affects steady-state and stress erythropoiesis, which is compensated by *Xpo7A* expression in blood, so next we evaluated the complete *Xpo7AB* KO mice.

Null Xpo7 results in partially penetrant embryonic lethality

$AB^{+/-}$ mice were fertile and indistinguishable from WT littermates. When heterozygous animals were intercrossed, we observed a significantly lower fraction of *Xpo7AB* KO offspring than the 25% predicted by Mendelian segregation (Table 1). Only 7% (5 of 72, $p<0.0001, \chi^2$) of postnatal progeny from $AB^{+/-}$ intercrosses were $AB^{-/-}$, suggesting that a significant number of homozygous knockout animals were lost during development.

To determine when during gestation AB KO embryos were lost, embryos from heterozygous intercrosses were isolated at blastocyst (E3.5) and mid-late gestation (E9.5-E15.5) stages of development. Knockout blastocysts appeared morphologically indistinguishable from their wild-type and heterozygous counterparts and were observed at the predicted Mendelian ratio of 25%. Analysis of embryos at mid-late gestation showed that *Xpo7AB* KO embryos were lost over a period between E11.5 and E13.5, when definitive erythropoiesis commences in the fetal liver of the mouse [34]. This time-specific loss of *Xpo7AB* KO embryos when definitive erythropoiesis begins suggests a critical role for Xpo7 in definitive erythropoiesis during murine fetal liver red cell development. To further understand the role of Xpo7 in *adult* steady state and stress erythropoiesis, we generated and evaluated inducible *Xpo7AB* KO mice as adults.

Xpo7 is important for stress erythropoiesis in vivo

To further understand the function of Xpo7 in adult erythropoiesis *in vivo*, we generated an inducible AB KO mouse model using the tamoxifen-inducible Cre recombinase system. The $AB^{fl/fl}$ floxed mice were born in a normal Mendelian distribution from heterozygous intercrosses. Mice at 4–5 weeks of age were injected with 1 mg/day of tamoxifen for 5 days. Since the RBC turnover period in mice is about 42 days, analysis of hematological parameters was performed 6 weeks after tamoxifen administration. Knockout mice displayed morphologically normal RBC (Figure 4A) and complete loss of Xpo7 in peripheral blood (Figure 4B), but showed mild anemia with statistically lower HCT, and higher RDW with normal spleen size when compared to controls (Figure 4C, **** = $p<0.0001$, ** = $p<0.001$, * = $p<0.01$, two-way ANOVA).

In order to examine stress erythropoiesis, 9–10 week-old male mice were treated once with 80 mg/kg/day of PHZ and we compared each mouse to itself after PHZ treatment. Impaired recovery following PHZ treatment was evident from the statistically lower RBC and HCT and higher RDW and retic count in KO mice compared to control (Figure 4D, **** = $p<0.0001$, ** = $p<0.001$, * = $p<0.01$, two-way ANOVA). To test recovery from even more severe hemolytic anemia, we injected 9–10 week-old mice with a single dose of 100 mg/kg/day of PHZ and found that fewer KO animals survived than control mice (Figure 4E). Analysis of bone marrow of these animals subject to a higher dose of PHZ showed impaired terminal erythroid differentiation with statistically fewer terminal orthochromatic erythroblasts in KO compared to WT BM even 14 days after PHZ administration (Figure 4F). The more severe hemolytic anemia resulted in comparable erythroblast populations in the spleens of KO and WT mice. In mice of both groups that did not survive beyond day 3, the percentage of reticulocytes in the BM of KO was significantly higher than that in WT and vice versa in case of the spleen (Supp Figure S3). Despite a high rate of reticulocytosis in the BM of KO mice, most of them fail to survive as a result of impaired stress erythropoiesis in the spleen (evident from the low reticulocytosis). The mutants also show a deficit in other erythroblast populations in the spleen.

Further, we see that the erythroid-specific isoform Xpo7B, as compared to the ubiquitous isoform Xpo7A, is specifically expressed in the erythropoietic organs of the WT mouse (Supp Figure S5). Taken together, this further highlights the significance of Xpo7 in stress erythropoiesis.

In summary, *Xpo7AB* knockout mice were born in decreased Mendelian ratios, and inducibly-knocked out adults showed mild anemia and higher RDW with normal spleen size. Knockouts had severely impaired response to stress erythropoiesis after phenylhydrazine treatment compared to controls and fewer terminal erythroblasts in KO compared to WT BM. These findings suggest that the stress erythropoietic response is impaired by the complete loss of Xpo7, so we evaluated the expression of known stress erythropoiesis regulators in the bone marrow of *Xpo7AB* KO animals to evaluate the mechanism.

Xpo7 loss downregulates the expression levels of Gdf15 and Smad3

To understand the impaired response to hemolytic anemia after loss of Xpo7, we examined the levels of known stress erythropoiesis regulators, Gdf15 [17] and Smad3, in the erythroid cells from the bone marrow of *Xpo7AB* KO and WT mice. As levels of these erythroid regulators are known to rise in response to anemia, we hypothesized that *Xpo7* KO mice would be unable to synthesize adequate levels of these regulators and hence fail to completely recover from hemolytic anemia. Consistent with our hypothesis, we observed a greatly diminished expression of both Gdf15 and Smad3 (Figure 5, $p<0.0001$, two-way ANOVA) in *Xpo7AB* KO mice when compared to WT. This suggests that there is a role of Xpo7 in pathways of stress erythropoiesis, specifically in the bone marrow response to stress, evident by the impaired increase of vital regulators Gdf15 and Smad3.

Discussion

Our group previously uncovered that the nuclear export protein Xpo7 is involved in terminal erythroid nuclear maturation[18]. Additionally, we found an unannotated erythroid-specific first exon (1b) of *Xpo7* exclusively expressed in Ter119+ erythroblasts but not in earlier erythroid progenitors (BFU-Es or CFU-Es), whereas the ubiquitous exon (1a) of *Xpo7* is expressed at modest levels in many tissues[18]. In this study, we examined the *in vivo* function of the two isoforms, Xpo7A and Xpo7B, using targeted mouse models. Given that Xpo7 is erythroid-specific, highly induced during terminal erythropoiesis, and is the only importin-B family member remaining during terminal erythroid development[18], as expected, loss of the nonerythroid isoform (Xpo7A) had no effect on erythropoiesis. In contrast, we showed that the erythroid-specific isoform of Xpo7 (Xpo7B) plays an important role in basal and stress erythropoiesis (Figure 3). We also demonstrated that complete Xpo7 loss led to a partially penetrant embryonic lethality, in which most homozygous null animals were lost at the embryonic stage marking the onset of fetal liver development (Table 1). Colony formation assays of fetal liver progenitors from *Xpo7*-null cells were normal, confirming that loss of Xpo7 does not affect erythroid cell fate (Supp Figure S1). Using an inducible model, we showed that the complete loss of Xpo7 in adult mice results in impaired response to hemolytic stress (Figure 4). Surprisingly, based on the images and measurement of enucleation via flow cytometry after culture of fetal liver cells, Xpo7 did not play a direct role in enucleation *ex vivo* and *in vivo* (Supp Figure S2). Given that no mammal survives to adulthood without proper enucleation (no transgenic mouse model exists with nucleated red cells in the adult), it is possible that the process is so essential for embryogenesis that other enucleation regulatory pathways may compensate for Xpo7 loss *in vivo*, a phenomenon not captured by *in vitro* experiments in our earlier published work [18]. This also reflects the differences in regulatory and signaling mechanisms *in vivo* due to presence of a highly coordinated hematopoietic niche when compared to monocultures *in vitro*. Given the much more significant phenotype of complete Xpo7 loss than Xpo7B loss (as defined from the significantly lower HCT in the inducible KO mice), we suggest that Xpo7A provides compensation of function, even though it is primarily expressed outside of the erythroid system at modest levels. Similarly, the lack of anemia in Xpo7A KO mice suggests that the erythroid-specific isoform Xpo7B may be compensating in recovery from

hemolysis. Instances of compensatory mechanisms as such are not uncommon in cases of genes with multiple isoform variants [35].

We examined further the role of Xpo7 in stress erythropoiesis by evaluating the expression profile of Xpo7 null cells, and specifically noted disrupted transcript levels of several established regulators of stress erythropoiesis. Our study uncovered a novel function for Xpo7 in erythropoiesis in the transcriptional regulation of the effectors Gdf15 and Smad3 (Figure 5). Recent findings have shown that Gdf15 signaling is indispensable in maintaining the stress erythropoiesis niche and subsequent recovery from hypoxic anemia [17, 36]. We noticed a delayed recovery following PHZ-induced hemolytic anemia in *Xpo7AB* KO mice, similar to that observed in $Gdf15^{-/-}$ mice[17]. Gdf15 signaling controls Bmp4 expression, which also regulates stress erythropoiesis in response to hypoxia. In response to TGF-β, a complex containing Smad3 stimulates erythroid differentiation[37]. Smad3 is also known to regulate Epo expression in response to hypoxia [38]. A recent study in human cervical epithelial cancer cells has shown that diminished expression of GDF15 decreased expression of p-Smad3 thereby affecting the activity of the TGF-β/SMAD signaling pathway [39]. While canonically Bmp4 acts via Smad1/5 [40] and there is no evidence of overlap of the pathways between Gdf15 and Smad3 in stress erythropoiesis, induction of *both* of these crucial regulators was impaired by loss of Xpo7. This evidence suggests a novel role for Xpo7 in recovery from hemolytic anemia. This is corroborated by downregulation of Xpo7 in the Klf1-*Nan* mutants, which display a form of congenital hemolytic anemia, as recently reported [41]. Perry et al [42] demonstrated that Hedgehog signaling is important for BMP4 induction which further drives stress erythropoiesis in murine spleen and a very recent independent study has shown that Xpo7 regulates Hedgehog signaling negatively [43]. This further reaffirms our findings of a delayed recovery from anemia in Xpo7 deficient animals due to inadequate stress erythropoiesis.

Targeted loss of Xpo7 and its isoforms reveals that Xpo7 plays a potential role in the recovery from hemolytic anemia and provides insight into novel transcriptional regulation of two crucial regulators of stress erythropoiesis. While Xpo7 has not been implicated in transcriptional regulation previously, there is precedence in other exporters such as Crm1 (Xpo1) that affect transcription by nucleocytoplasmic control of transcriptional modulators [44]. This underlies the need for further detailed investigation of additional functions of Xpo7. This intriguing hypothesis is supported by the fact that Xpo7 was found to be a direct binding partner of Smad3 as detected by affinity chromatography[45]. In addition to playing an important role in terminal erythroid nuclear maturation by facilitating the export of histones to the cytoplasm[18], our work has identified a new role for Xpo7 in stress erythropoiesis as a novel transcriptional regulator of the stress erythropoiesis regulators Gdf15 and Smad3. These data warrant further study of the direct biochemical function of Xpo7, which we are currently performing using constructs of Xpo7A and B in *ex vivo* culture of fetal liver erythroid progenitors from *Xpo7*-null mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by the NIDDK R01 DK102889 (SH), the Rudolph Anderson Fellowship (Yale Medical School) (SM), and U54 (GMK and SH). GMK is supported by an ASH Bridge Award grant and NIDDK R01 DK111539.

The authors would also like to acknowledge the following people for their contribution:

Tim Nottoli, Xiaojun Xing from Yale Genome Editing Center,

Nadia Ayala-Lopez, Betty Lawton (Krause Lab, Yale University), Samuel Oguntayo (DCM, Georgetown University): blood smears, embryo dissection material

Rithy Meas (Sweasy Lab, Yale University): dissection microscope

Lin Lab (Stem Cell Center, Yale University), Peter Johnson (Microscopy and Imaging resource, Georgetown University): microscope for blood smears

References

1. NE. D, Erythropoiesis. 10th ed. Vol. 1. 1999, Baltimore: Williams & Wilkins.
2. Ji P, Murata-Hori M, and Lodish HF, Formation of mammalian erythrocytes: chromatin condensation and enucleation. Trends Cell Biol, 2011. 21(7): p. 409–15. [PubMed: 21592797]
3. Hosoya T, et al. , TRIM28 is essential for erythroblast differentiation in the mouse. Blood, 2013. 122(23): p. 3798–807. [PubMed: 24092935]
4. Thom CS, et al. , Trim58 degrades Dynein and regulates terminal erythropoiesis. Dev Cell, 2014. 30(6): p. 688–700. [PubMed: 25241935]
5. Rouzbeh S, et al. , Molecular signature of erythroblast enucleation in human embryonic stem cells. Stem Cells, 2015. 33(8): p. 2431–41. [PubMed: 25850942]
6. Zhang L, et al. , miR-191 regulates mouse erythroblast enucleation by down-regulating Riok3 and Mxi1. Genes Dev, 2011. 25(2): p. 119–24. [PubMed: 21196494]
7. Zhao B, et al. , Nuclear Condensation during Mouse Erythropoiesis Requires Caspase-3-Mediated Nuclear Opening. Dev Cell, 2016. 36(5): p. 498–510. [PubMed: 26954545]
8. Li X, et al. , Histone deacetylase 6 regulates cytokinesis and erythrocyte enucleation through deacetylation of formin protein mDia2. Haematologica, 2017. 102(6): p. 984–994. [PubMed: 28255013]
9. Gnanapragasam MN, et al. , EKLF/KLF1-regulated cell cycle exit is essential for erythroblast enucleation. Blood, 2016. 128(12): p. 1631–41. [PubMed: 27480112]
10. Aoto M, et al. , Transferrin receptor 1 is required for enucleation of mouse erythroblasts during terminal differentiation. FEBS Open Bio, 2019. 9(2): p. 291–303.
11. Goto T, et al. , ATP produced by anaerobic glycolysis is essential for enucleation of human erythroblasts. Exp Hematol, 2019. 72: p. 14–26 e1. [PubMed: 30797950]
12. Socolovsky M, Molecular insights into stress erythropoiesis. Current opinion in hematology, 2007. 14(3): p. 215–224. [PubMed: 17414210]
13. Paulson RF, Shi L, and Wu D-C, Stress erythropoiesis: new signals and new stress progenitor cells. Current opinion in hematology, 2011. 18(3): p. 139. [PubMed: 21372709]
14. Lenox LE, Perry JM, and Paulson RF, BMP4 and Madh5 regulate the erythroid response to acute anemia. blood, 2005. 105(7): p. 2741–2748. [PubMed: 15591122]
15. Lenox LE, et al. , Extramedullary erythropoiesis in the adult liver requires BMP-4/Smad5–dependent signaling. Experimental hematology, 2009. 37(5): p. 549–558. [PubMed: 19375646]
16. Porayette P.and Paulson RF, BMP4/Smad5 dependent stress erythropoiesis is required for the expansion of erythroid progenitors during fetal development. Developmental biology, 2008. 317(1): p. 24–35. [PubMed: 18374325]
17. Hao S, et al. , Gdf15 regulates murine stress erythroid progenitor proliferation and the development of the stress erythropoiesis niche. Blood advances, 2019. 3(14): p. 2205–2217. [PubMed: 31324641]

18. Hattangadi SM, et al. , Histones to the cytosol: exportin 7 is essential for normal terminal erythroid nuclear maturation. Blood, 2014. 124(12): p. 1931–40. [PubMed: 25092175]
19. Figueroa AA, et al. , miR-181a regulates erythroid enucleation via the regulation of Xpo7 expression. Haematologica, 2018. 103(8): p. e341–e344. [PubMed: 29567782]
20. Schwenk F, Baron U, and Rajewsky K, A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. Nucleic Acids Res, 1995. 23(24): p. 5080–1. [PubMed: 8559668]
21. Ventura A, et al. , Restoration of p53 function leads to tumour regression in vivo. Nature, 2007. 445(7128): p. 661–665. [PubMed: 17251932]
22. Santos EW, et al. , Hematological and biochemical reference values for C57BL/6, Swiss Webster and BALB/c mice. Brazilian Journal of Veterinary Research and Animal Science, 2016. 53(2): p. 138–145.
23. Provencher Bolliger A, et al., Hematology of laboratory animals, p 852–887. Chapter 110. Schalm's Veterinary Hematology, 6th ed. Ames (IA): Wiley-Blackwell, 2010.
24. Zhang L, et al. , ZFP36L2 is required for self-renewal of early burst-forming unit erythroid progenitors. Nature, 2013. 499(7456): p. 92–6. [PubMed: 23748442]
25. Bogacheva O, et al. , DYRK3 dual-specificity kinase attenuates erythropoiesis during anemia. J Biol Chem, 2008. 283(52): p. 36665–75. [PubMed: 18854306]
26. Flygare J, et al. , HIF1alpha synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. Blood, 2011. 117(12): p. 3435–44. [PubMed: 21177435]
27. Hattangadi SM, Burke KA, and Lodish HF, Homeodomain-interacting protein kinase 2 plays an important role in normal terminal erythroid differentiation. Blood, 2010. 115(23): p. 4853–61. [PubMed: 20231426]
28. Zhang J, et al. , Role of Ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. Blood, 2003. 102(12): p. 3938–46. [PubMed: 12907435]
29. Chen K, et al. , Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci U S A, 2009. 106(41): p. 17413–8. [PubMed: 19805084]
30. Dev A, et al. , During EPO or anemia challenge, erythroid progenitor cells transit through a selectively expandable proerythroblast pool. Blood, 2010. 116(24): p. 5334–46. [PubMed: 20810925]
31. Livak KJ and Schmittgen TD, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. Methods, 2001. 25(4): p. 402–8. [PubMed: 11846609]
32. Lee H-Y, et al. , PPAR-α and glucocorticoid receptor synergize to promote erythroid progenitor self-renewal. Nature, 2015. 522(7557): p. 474–477. [PubMed: 25970251]
33. Dumitriu B, et al. , Sox6 is necessary for efficient erythropoiesis in adult mice under physiological and anemia-induced stress conditions. PLoS One, 2010. 5(8): p. e12088. [PubMed: 20711497]
34. Palis J, Primitive and definitive erythropoiesis in mammals. Frontiers in physiology, 2014. 5: p. 3. [PubMed: 24478716]
35. El-Brolosy MA and Stainier DY, Genetic compensation: A phenomenon in search of mechanisms. PLoS genetics, 2017. 13(7): p. e1006780. [PubMed: 28704371]
36. Larissi K, et al. , The Growth Differentiation Factor-15 (GDF-15) levels are increased in patients with compound heterozygous sickle cell and beta-thalassemia (HbS/beta(thal)), correlate with markers of hemolysis, iron burden, coagulation, endothelial dysfunction and pulmonary hypertension. Blood Cells Mol Dis, 2019. 77: p. 137–141. [PubMed: 31071550]
37. He W, et al. , Hematopoiesis controlled by distinct TIF1γ and Smad4 branches of the TGFβ pathway. Cell, 2006. 125(5): p. 929–941. [PubMed: 16751102]
38. Zhang Y, et al. , Erythropoietin Action in Stress Response, Tissue Maintenance and Metabolism. International Journal of Molecular Sciences, 2014. 15(6): p. 10296–10333. [PubMed: 24918289]
39. Li L, et al. , GDF15 knockdown suppresses cervical cancer cell migration in vitro through the TGF β/Smad2/3/Snail1 pathway. FEBS Open bio, 2020. 10(12): p. 2750–2760.

40. Dituri F, et al. , The Interactivity between TGFβ and BMP Signaling in Organogenesis, Fibrosis, and Cancer. Cells, 2019. 8(10): p. 1130. [PubMed: 31547567]

41. Cantú I, et al. , The mouse KLF1 Nan variant impairs nuclear condensation and erythroid maturation. PLOS ONE, 2019. 14(3): p. e0208659. [PubMed: 30921348]

42. Perry JM, et al. , Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. Blood, The Journal of the American Society of Hematology, 2009. 113(4): p. 911–918.

43. Markiewicz Ł, et al. , Xpo7 negatively regulates Hedgehog signaling by exporting Gli2 from the nucleus. Cellular Signalling, 2021. 80: p. 109907. [PubMed: 33383157]

44. Guillem F, et al. , XPO1 regulates erythroid differentiation and is a new target for the treatment of β-thalassemia. Haematologica, 2019.

45. Aksu M, et al. , Xpo7 is a broad-spectrum exportin and a nuclear import receptor. Journal of Cell Biology, 2018. 217(7): p. 2329–2340. [PubMed: 29748336]

Highlights

- Novel role of *Xpo7* in recovery from hemolytic anemia in normal and stress erythropoiesis
- Loss of erythroid-specific isoform of *Xpo7* causes anemia comparable to that of complete Xpo7 loss
- Loss of Xpo7 downregulates expression of key factors involved in the erythroid stress response pathway (Gdf15, Smad3).

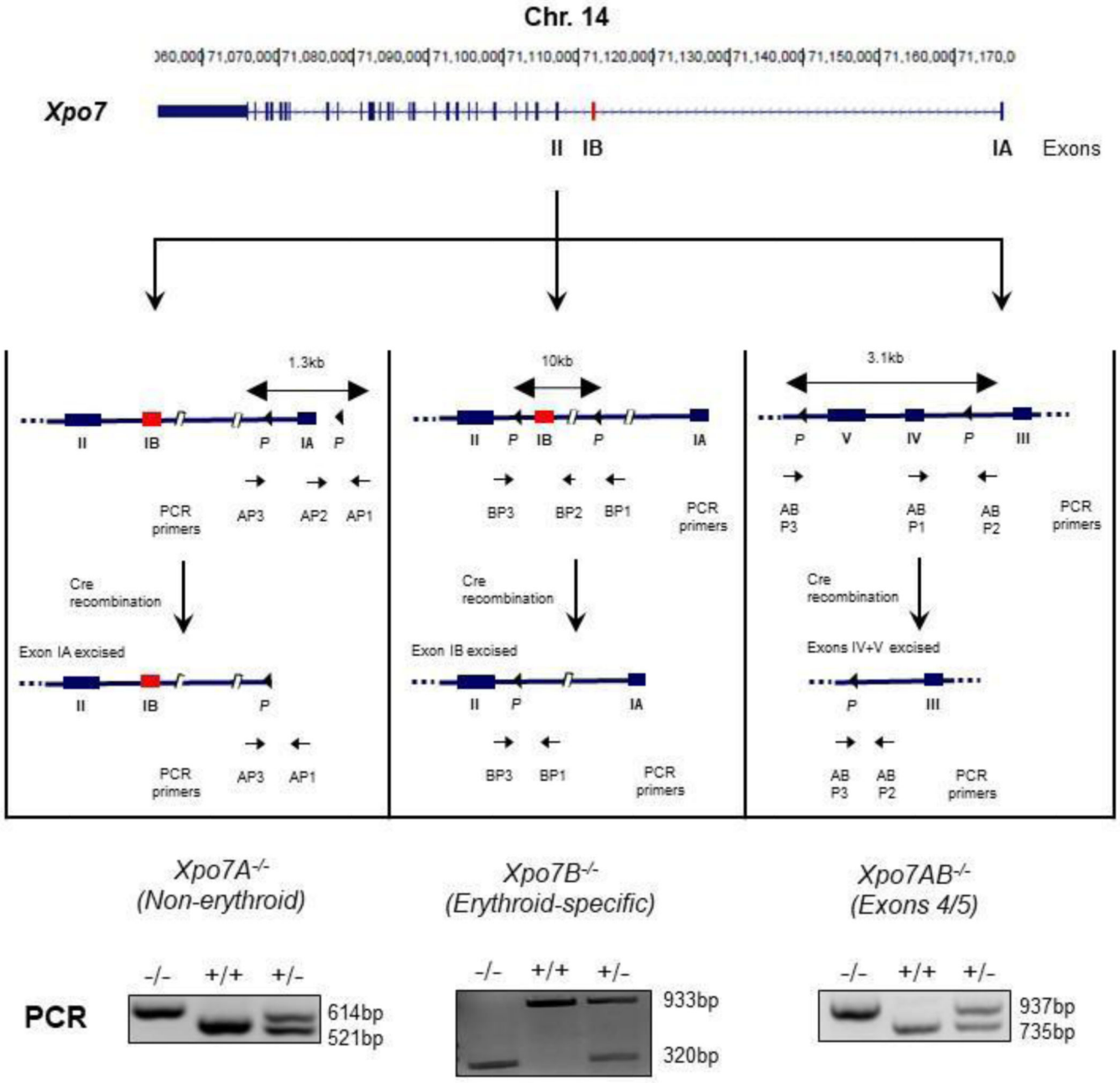

Figure 1. Mice lacking either isoforms Xpo7A or B or both were generated.

Top, the mouse *Xpo7* gene. Middle, floxed *Xpo7* allele generated by inserting 2 *loxP* sites surrounding exon 1A (non-erythroid specific), 1B (erythroid-specific) and exons 4+5, respectively. Bottom, *Xpo7* null allele obtained after Cre-mediated recombination and PCR for KO (−/−), WT (+/+) and HET (+/−) animals.

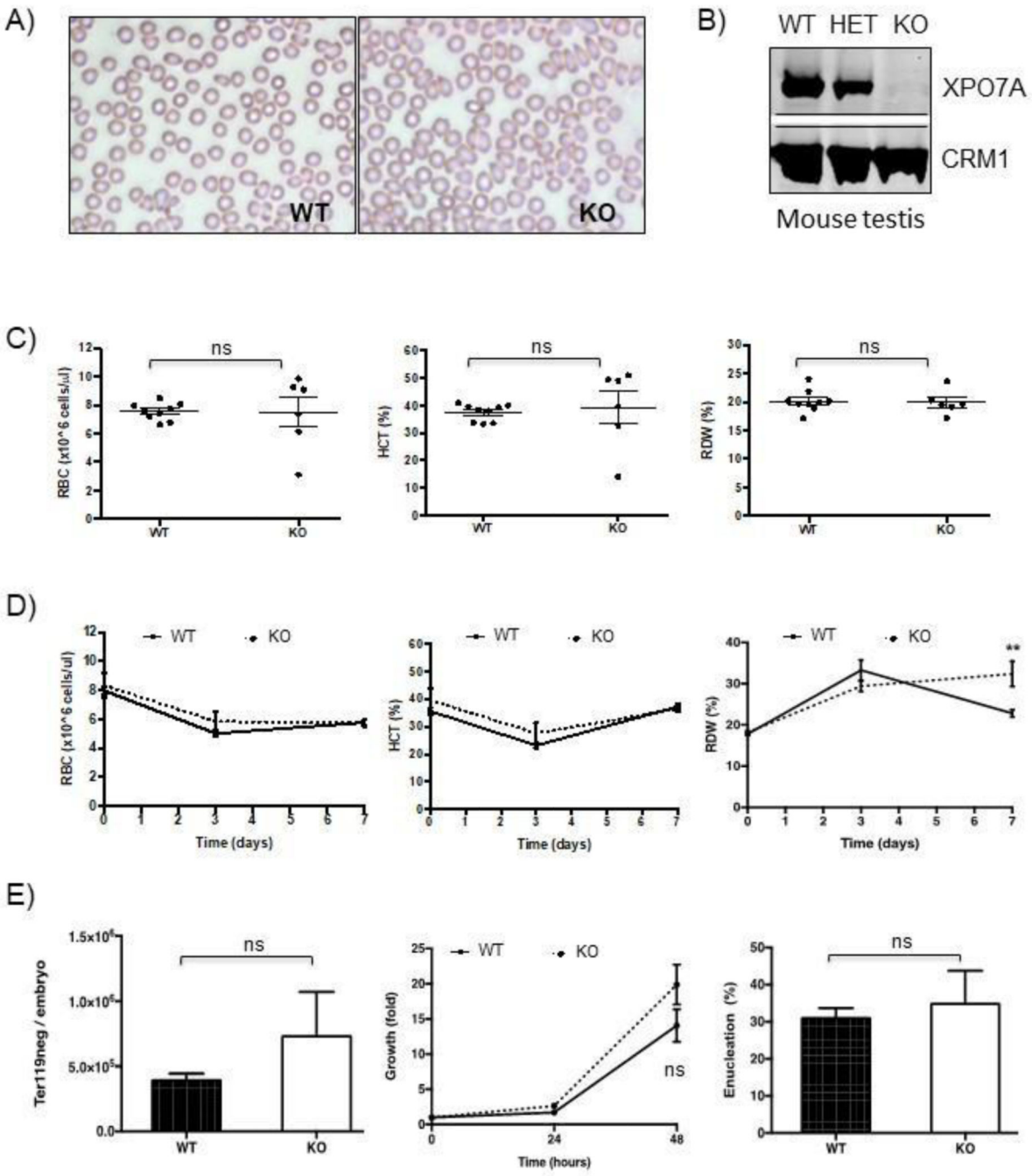

Figure 2. The ubiquitous isoform of *Xpo7* gene, *Xpo7A*, is not essential for erythropoiesis.

(A) Representative pictures of blood smears of WT and KO *Xpo7A*$^{-/-}$ mice (magnification 64X). (B) express blot validating the KO of Xpo7A (in mouse testis where it is most prominent). (C) Peripheral blood counts of *Xpo7A*$^{-/-}$ and control mice at 5 weeks age. Results are shown as mean ± SEM from 6 – 9 mice per group (**** = p<0.0001, ** = p<0.001, * = p<0.01, two-way ANOVA): red blood cell count (RBC), hematocrit (HCT) and red cell distribution width (RDW). (D) Animals were treated with PHZ during two consecutive days. RBC, HCT and RDW were measured on the days indicated (n = 6 – 9 per

group). (E) Ter119neg cells isolated from fetal livers of WT and KO embryos (n = 4–6 per group) were counted and cultured *in vitro* for two days. Cell growth (fold) and enucleation (% of reticulocytes, Ter119+/Hoechst 33342-) were assessed.

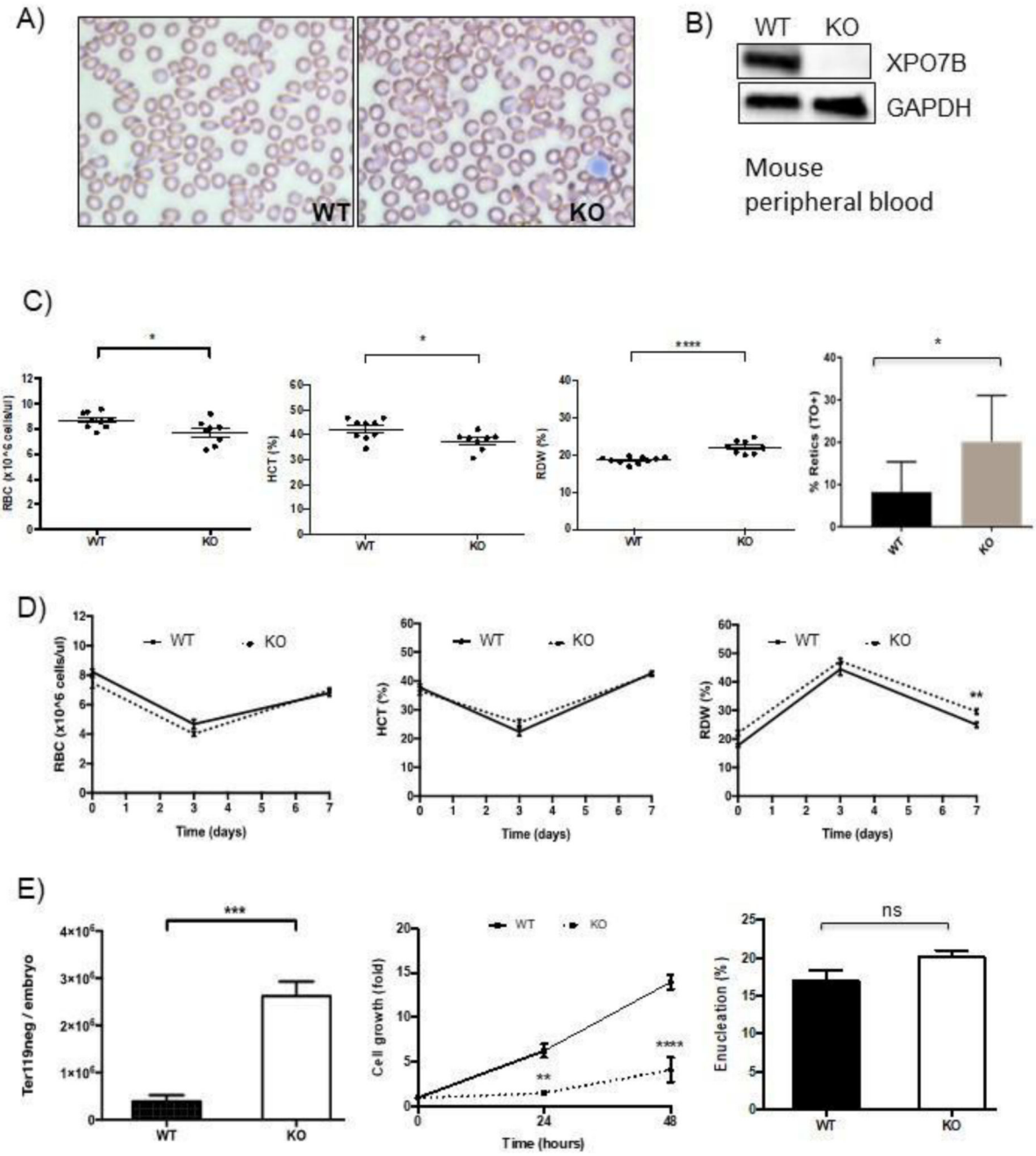

Figure 3. Mice lacking the erythroid-specific isoform of *Xpo7, Xpo7B*, show a mild anemia and altered stress erythropoiesis.

(A) Representative pictures of blood smears of WT and KO *Xpo7B*$^{-/-}$ mice (magnification 64X). (B) Western blot validating the KO of *Xpo7B*. (C) Peripheral blood counts of *Xpo7B*$^{-/-}$ and control mice at 5 weeks age. Results are shown as mean ± SEM from 8 – 9 mice per group (**** = p<0.0001, ** = p<0.001, * = p<0.01, two-way ANOVA): RBC, HCT, RDW and Reticulocytes. (D) Animals were treated with PHZ during two consecutive days. RBC, HCT and RDW were measured on the days indicated (n = 8 – 9 per group). (E) Ter119neg cells isolated from fetal livers of WT and KO embryos (n = 5 – 7 per group)

were counted and cultured *in vitro* for two days. Cell growth (fold) and enucleation (% of reticulocytes, Ter119+/Hoechst 33342−) were assessed.

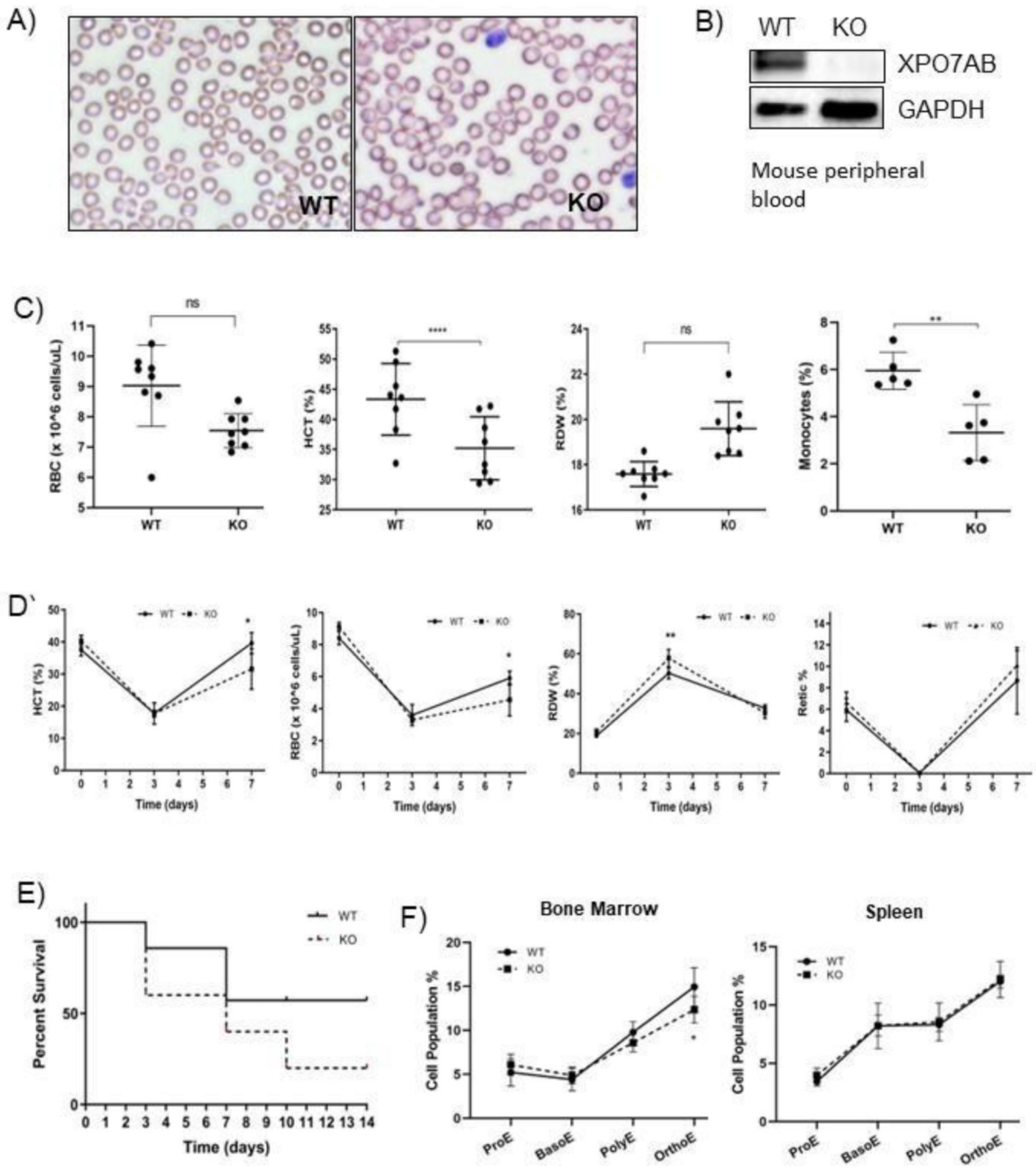

Figure 4. Complete Xpo7 deficiency impairs both steady-state erythropoiesis and stress erythropoiesis.

(A) Representative pictures of blood smears of WT and KO of *Xpo7AB*$^{-/-}$ mice (magnification 64X). (B) Western blot validating the KO of complete *Xpo7*. (C) Peripheral blood counts of *Xpo7AB*$^{-/-}$ and control mice at 5 weeks age. Results are shown as mean ± SEM from 8 mice per group (**** = p<0.0001, ** = p<0.001, * = p<0.01, two-way ANOVA): HCT and RDW. (D) Animals were treated with PHZ (80mg/kg/day) once. RBC, HCT, RDW and reticulocytes were measured on the days indicated (n = 5 – 6 per group). (E) Survival curve of WT and KO animals following treatment with PHZ (100mg/kg/day). (F)

Percentage of erythroblast populations in bone marrow and spleen of WT and KO animals following recovery from stress erythropoiesis.

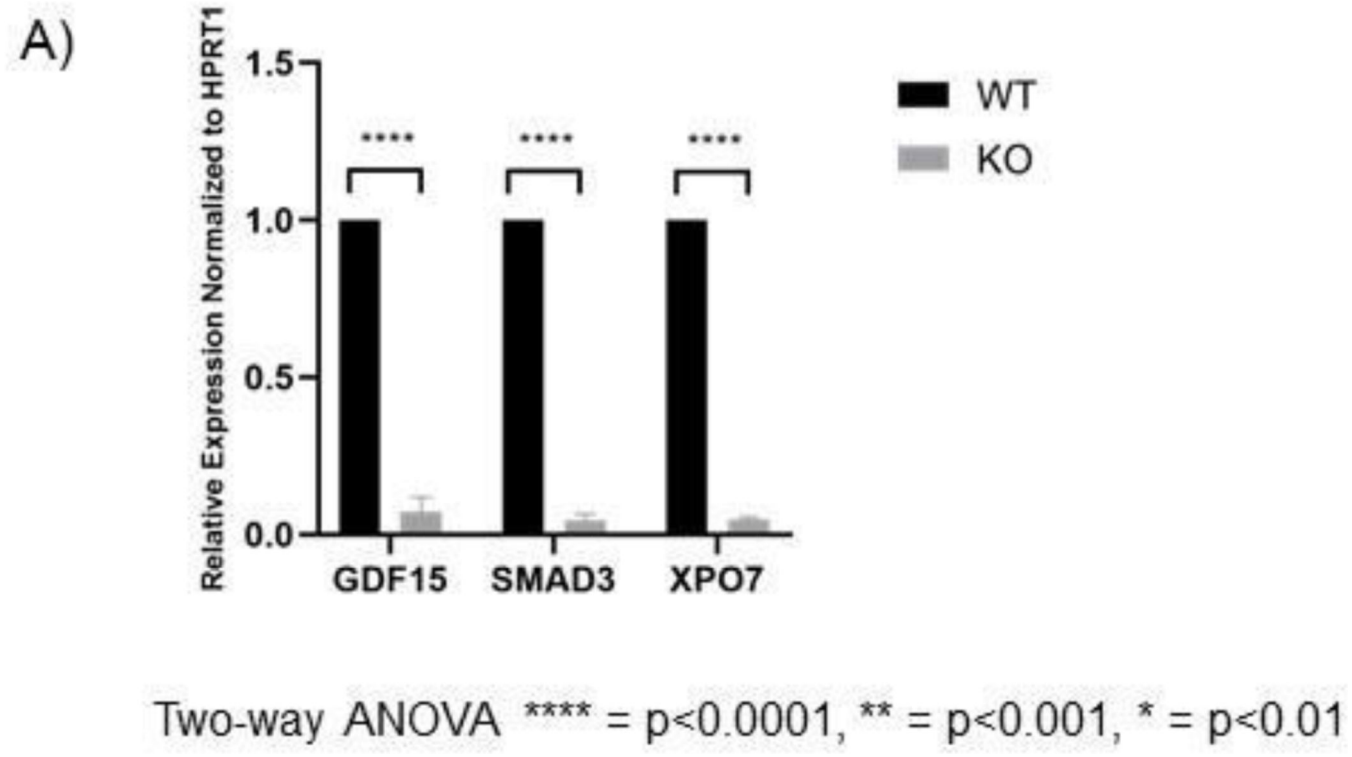

Figure 5. Xpo7 loss inhibits expression of stress erythropoiesis regulators Gdf15 and Smad3.

(A) Quantitative PCR (qPCR) analyses of Gdf15 and Smad3 from the BM of *Xpo7* KO and control mice. Analyses were performed in replicates of 3. Data were normalized with hypoxanthine phosphoribosyltransferase-1 (Hprt1) mRNA levels. Results are shown as mean ± SEM (error bars), **** = p<0.0001, ** = p<0.001, * = p<0.01, two-way ANOVA.

Table 1.

Constitutive total Xpo7 deficiency in mice results in partially penetrant embryonic lethality.

				Genotypes (%)		
Mouse strain		**Stage**	**Total**	**WT**	**Het**	**KO**
Xpo7AB−/−		E3.5	14	4 (29)	3 (21)	7 (50)****
		E9.5	18	6 (33)	8 (45)	4 (22)^{ns}
		E11.5	16	2 (13)	9 (56)	5 (31)****
		E13.5	29	14 (48)	11 (38)	4 (14)**
		E15.5	20	8 (40)	7 (35)	5 (25)**
		Postnatal	72	26 (36)	41 (57)	5 (7)**
Xpo7A−/−		Postnatal	66	29 (44)	27 (41)	10 (15)^{ns}
Xpo7B−/−		Postnatal	36	6 (17)	17 (56)	10 (27)^{ns}